

Review

Fish tales: The use of zebrafish xenograft human cancer cell models

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Summary. Advances in scientific techniques have provided researchers with exceptional new opportunities to identify and monitor changes between different cancer types, during different stages of progression, between individual tumor cells and in the surrounding stroma. The wealth of information that can be obtained from new scientific techniques places additional requirements on the conventional cancer models. New models that could be used to rapidly access the (potential) functional importance of newly identified (epi)genetic and proteomic changes and test the efficacy on emerging (combinatorial) therapies are desperately required. The distinctive characteristics of zebrafish are progressively being applied to create more relevant models of human diseases. Zebrafish embryos provide a powerful tool to develop functional cancer models. This is a tool that can be used from drug discovery and development to assessment of drug toxicity. This review will summarise the use of zebrafish xenograft models to study human cancers, and discuss the benefits and limitations of these models.

Key words: Cancer, Drug screening, Embryo, Xenograft, Zebrafish

Introduction

Cancer is a genetic disease (Hanahan and Weinberg, 2011). Cancer is a prominent cause of human morbidity and mortality (McGuire, 2016). Researchers face a great challenge in trying to understand the (epi)genetic basis of this disease, the role of tumor heterogeneity and the interactions between stroma and tumor cells, the mechanisms that underlie why certain patients do or do not respond to therapy, and how to prevent therapy resistance. In order to study the biochemical pathways in cancer cells, researchers use animal and human cancer cell lines *in vitro* and *in vivo*. The most common *in vivo* cancer model is that of the rodent, specifically the mouse: 95% of its genetic material is similar to humans (Mouse Genome Sequencing et al., 2002). There are many short-comings to the mouse model and a search is on to find alternatives. One such *in vivo* model is the zebrafish (*Danio rerio*), which is proving to be an elegant model to study human cancer.

Murine models have revolutionised the study of gene and protein function *in vivo*. The mouse model allowed for the greater understanding of the molecular pathways and mechanisms used by cancer cells (Frese and Tuveson, 2007). New developments, such as organoids, can allow for the testing of drugs on human cancer cells in a mouse background (Sachs and Clevers, 2014). However, these studies need to be done in immune-compromised mice, which do not allow for efficient testing of immune system in the treatment response. Moreover, testing of many (combinations of) (epi)genetic changes and drugs on tumor initiation and

progression quickly is difficult to archive in a mouse.

What would be the ideal model for human cancer? Simply put, a model that perfectly reflects the tumor and its microenvironment. The criteria of an ideal model includes the need to imitate the human tumor clonal origin, as well as the histopathology of the tumor, and the various stages of tumorigenesis (including metastasis and recurrence). In addition, the ideal model should be able to accommodate several mutations in specific genes as well as alterations in specific pathways that are known to be involved in human cancers and the genomic instability that induces gross chromosomal aberrations. This ideal should be useful in the testing of new therapies and the identification of biomarkers. Moreover, this model should be able to be used in high-throughput screening. Furthermore, the drug response in the model should accurately anticipate the results of clinical trials (Hann and Balmain, 2001). Finding one model that encompasses all of these traits will be impossible. However, a model does not need to be to completely faithful to the human disease equivalent for it to be effective to address one particular aspect. For example, in basic cancer research a simple model that is suitable for high-throughput screening may be a better tool than a highly complex model that contains parameters that are not utilised.

While the initial focus of zebrafish research was in the field of developmental biology (Grunwald and Eisen, 2002), zebrafish made their debut splash as a cancer model in the 1960s (Stanton, 1965). Although the last common ancestor of fish and human is separated by more than 400 million years, the biology of cancer in these two organisms share many characteristics (Schartl, 2014), both at the molecular level (Abouheif et al., 1997; Dodd et al., 2000; Woods et al., 2000) and physiologically (Lieschke et al., 2001; Weinstein, 2002; Guyon et al., 2007; Kalueff et al., 2014). In addition, the architecture and microenvironment of a tumor can be live monitored in zebrafish cancer systems (Ignatius and Langenau, 2011). Zebrafish are prolific breeders, which reach sexual maturity by 3 months. A lone female can produce up to 100 eggs in one session, and the eggs are fertilized externally. They have a short life cycle; their major organs are fully developed by 24 hours post fertilization (HPF) and the larvae can be ready for experimental use by 3 days post fertilization (DPF). The embryos are transparent though to 7 DPF, and this can be prolonged up to 14 DPF with the use of melanin synthesis inhibitors (White et al., 2008). Zebrafish are also small and do not require expensive food – they can be cost effective. It is no surprise that zebrafish are considered a strong model system for the study of human disease (Lieschke and Currie, 2007; Pyati et al., 2007). This is especially true for zebrafish embryos, as they have an immature immune system, can be injected with human tumor cells, where tumor growth, invasion and metastasis, and the interplay with host cells can be easily examined.

This review will summarize the use of zebrafish xenografts with human tumor cells. This review will

discuss the advances of genetic manipulation of the fish to investigate different interactions of cancer cells, for example with the microenvironment such as angiogenesis and immune cells. We will also highlight the different xenotransplantation approaches. Finally, some of the benefits and limitations of using zebrafish xenograft models will be discussed. This review will not discuss spontaneous, drug induced, or transgenic cancer models. Reviews that do cover these topics have been authored by the Zon (White et al., 2013), and White (White, 2015) groups.

Xenotransplantation

Xenotransplantation involves the transfer (transplantation, implantation, or infusion) of one species-specific tissue to another animal species. This procedure has been used for many years in the study of human cancer (Sharkey and Fogh, 1984; Cekanova and Rathore, 2014). Xenograft experiments are commonly done in mouse models, and have enabled the examination of cancer cell proliferation, invasion (Sanchez-Tillo et al., 2012), migration (van Marion et al., 2016) and induction of tumor angiogenesis (Carmeliet and Jain, 2000). Professor Edward Sausville, has been quoted (Garber, 2006) as saying that “mouse xenografts will remain the ‘gold standard’ in cancer drug development.” But given restraints of time and cost, as well as the complexity of a mouse system, it means that it is not always practical to use mice.

Zebrafish present a unique opportunity to extend the knowledge gained from xenograft models. Zebrafish - human xenotransplantation models are being utilized to investigate a number of critical facets in tumor biology, comprised of cancer cell growth (Haldi et al., 2006), invasion (Naber et al., 2013; Yang et al., 2013), induction of angiogenesis (Tobia et al., 2011; He et al., 2012), metastasis (He et al., 2012; Drabsch et al., 2013; van der Ent et al., 2014a), and identification of new treatment strategies with translational values (Goessling et al., 2007; Konantz et al., 2012; Ghotra et al., 2015; Zoni et al., 2015; Mercatali et al., 2016). Most of these investigations injected human cancer cells into early-stage zebrafish embryos. This is due to 3 main considerations:

- 1) The adaptive immune system of a zebrafish has not developed until 14 DPF (Renshaw and Trede, 2012). The human cancer cells are thus able to survive, invade, and metastasize when transferred into the embryonic zebrafish.

- 2) The transplanted cells in the transparent embryonic zebrafish can be visualized easily (Ignatius and Langenau, 2011). This is due to the use of fluorescent labels.

- 3) Human cells can communicate with the zebrafish host (Tulotta et al., 2016). The intercommunication between the cells of these two species is conserved.

There are numerous sites of injection which have been used regularly in the zebrafish cancer model (Fig.

Zebrafish as cancer cell models

1). These sites include the yolk sac, the Duct of Cuvier and perivitelline space, and the hindbrain ventricle.

Site of injection: Yolk sac

The yolk sac is an environment rich in nutrients and suitable for the injected cells. Furthermore the yolk sac is large and acellular, and the injected cells can grow, migrate (if the cells are motile), and be easily monitored post injection. These considerations have meant that the most common site of injection is the yolk sac of 2 days post fertilization (DPF) zebrafish (Veinotte et al., 2014).

Researchers are using the yolk sac in more complex ways than previously. Recent research injected a mix of cancer cells and macrophages into the yolk sac of a zebrafish embryo. This study showed that previous exposure of macrophages to interleukin-6 (IL-6) and tumor necrosis factor (TNF) enhances the metastatic spread (Wang et al., 2015).

Due to the unique properties of the zebrafish embryo, it has allowed researchers to research human hematopoietic stem cell trafficking *in vivo* (Staal et al., 2016). The Staal group used the Casper *tg(fli-GFP)* (described in further detail below) as recipients of human CD34(+) red labelled cells. They successfully visualized the differentiation of the transplanted human hematopoietic stem cell CD34(+) cells, as well as tracking them within the vasculature of the fish.

Site of injection: Perivitelline space and the duct of cuvier

Though the yolk sac is widely used for xenografts in zebrafish, many groups prefer to study the later stages of cancer progression by injecting the cancer cells into either the perivitelline space (Nicoli and Presta, 2007), or into the circulation via the Duct of Cuvier (He et al., 2012; Zhang et al., 2012a,b; Drabsch et al., 2013; Naber et al., 2013; de Boeck et al., 2016). Whereas injection of fluorescently labelled cells in the perivitelline space allows one to interrogate the efficiency of intravasation, the entry of tumor cells directly into the blood

circulation allows the study of invasion and micrometastasis (He et al., 2012). Dependent on the cell line, different types of invasion (single cell or collective) can be easily visualised. This is because the cells invade into the tail fin. This xenograft model has contributed to understanding how the transforming growth factor β (TGF- β) pathway participates in the promotion of invasion and metastasis of human tumor cells (Drabsch and Ten Dijke, 2012; Zhang et al., 2012a,b; Drabsch et al., 2013; Naber et al., 2013; de Boeck et al., 2016). Furthermore, injecting into the perivitelline space was used to demonstrate vascularization of the tumor, localized tumour growth, and the involvement of neutrophils and macrophages at the site of implantation and the micrometastatic region (He et al., 2012).

This method of xenotransplantation offers an extraordinary prospect to live image the behaviour of human cancer cells and their interaction with the innate immune system *in vivo* (He et al., 2012; van der Ent et al., 2014b; Tulotta et al., 2016). Although this approach does not exactly recapitulate the initiation stages of a human tumour, they do represent aspects of the invasive spread of cancer, before and after the cancer cells have entered the circulation.

Site of injection: Hindbrain ventricle

There have been very few papers that have used the hindbrain as a site for injection. Research done by Haldi et al injected fluorescently labelled human melanoma cells into the hindbrain ventricle of 2dpf zebrafish (Haldi et al., 2006). This study demonstrated that masses from melanoma cells participated in the process of angiogenesis. Another study which used the hindbrain as an injection site focused on glioblastoma - a highly aggressive brain cancer. Glioblastoma cells implanted into the yolk sac did display an invasive phenotype. Interestingly, it was only when the glioblastoma cells were injected into the hindbrain ventricle these cells started to invade. This study makes evident that the location of xenografts is an important factor when

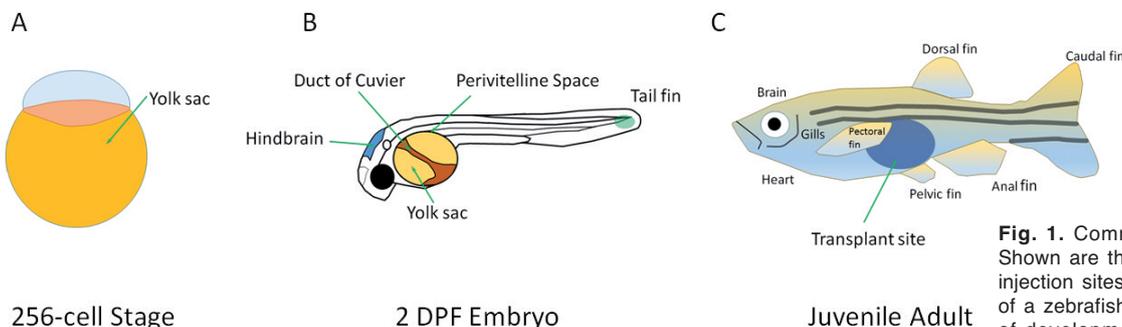


Fig. 1. Common Sites of Injection. Shown are the most commonly used injection sites for xenotransplantation of a zebrafish during different stages of development. **A.** 256-cell Stage.

The yolk sac (yellow) is the most common site of injection. **B.** 2 DPF zebrafish. The yolk sac (yellow) is the most common site for injection. This is followed by the Duct of Cuvier (orange) and perivitelline space and the hindbrain (blue). The site of invasion in the tail fin is highlighted in green. **C.** Juvenile Adult. The majority of xenografts occur within the intraperitoneal cavity.

designing *in vivo* experiments (Wehmas et al., 2016).

Adult zebrafish

The studies so-far highlighted in this review have used embryonic zebrafish hosts, and exploit the lack of an adaptive immune system, thus avoiding rejection. However, because of a recently created immunocompromised Rag2 mutant line, *rag2^{E450fs}* (Tang et al., 2014) use of the adult zebrafish is now available for xenograft studies. This transgenic line of fish is comparable to Rag-deficient mice. The *rag2^{E450fs}* zebrafish has diminished amount of T cells and B cells, and the adult *rag2^{E450fs}* zebrafish has been shown to be a universal recipient allograft cell transplantation.

Adult transparent zebrafish are used extensively in live *in vivo* imaging. For instance, to assess the pharmacological effects of a small molecule MAP kinase inhibitor (U0126) that targets the KRAS signalling pathway *in vivo*, human pancreatic cancer cells were xeno-transplanted into larval and adult zebrafish (Guo et al., 2015). KRAS mutant cells displayed substantial growth and migratory behaviour and invaded the vasculature system of adult zebrafish. U0126 treatment drastically reduced the proliferation and migration of the cells. Also in adult zebrafish, xenografted leukaemia stem cells (LSCs) from K562 cells were shown to proliferate *in vivo* and after re-transplantation kept their cancer

properties (Zhang et al., 2014). This is important as the current best method for defining a cancer stem cell is by exhibiting a cell's capacity to reconstitute a tumour *in vivo* by serial transplantation (Baccelli and Trumpp, 2012).

The results from these studies advocate the proposal that xenotransplantation in zebrafish is a simple and efficient tool to study a wide range of human cancers.

Zebrafish lines and development

An important development that could be partnered with xenotransplantation in order to research cancer cell progression in a living environment is fluorescent transgenic zebrafish reporter lines. Thousands of zebrafish reporter lines have been made and may be directly requested from the laboratories which generated them. Another way to obtain a specific zebrafish line is via public stock centres such as the Zebrafish International Resource Center (ZIRC; <http://zebrafish.org/zirc/home/guide.php>) and the European Zebrafish Resource Center (EZRC; <http://www.ezrc.kit.edu/>).

Some of the most widely used fluorescent reporter lines available are included in Table 1. Of particular importance are the *tg(fli1a-eGFP)*, *tg(mpx:eGFP)*, and *tg(mpeg1:eGFP)*. The *tg(fli1a-eGFP)* (Lawson and Weinstein, 2002) has been widely used since its inception in 2002. The *fli1* promoter drives enhanced GFP (eGFP) in endothelial cells. The zebrafish line

Table 1. Frequently used fluorescent reporter transgenic zebrafish lines.

| Zebrafish name | Site of expression | Reference |
|--|---|---|
| <i>tg(0.8flt1:TFP)hu5333</i> | arterial ISV | Bussmann and Schulte-Merker, 2011; Bussmann et al., 2011 |
| <i>tg(-1696a1T:GFP)</i> | glia cells | Goldman et al., 2001; Fausett et al., 2008 |
| <i>tg(5xUAS:cdh5-eGFP)</i> | endothelial cells | Lenard et al., 2013 |
| <i>tg(-7.8gata4:GFP)ae3</i> | endocardial and myocardial cells | Heicklen-Klein and Evans, 2004; Heicklen-Klein et al., 2005 |
| <i>tg(acta2:mCherry)</i> | vascular smooth muscle cells | Whitesell et al., 2014; Cetinkaya et al., 2016 |
| <i>tg(Brn3c:mGFP)</i> | dendritic cells | Xiao et al., 2005 |
| <i>tg(dll4:eGFP)</i> | arterial cells | Sacilotto et al., 2013 |
| <i>tg(dlx2b:EGFP)</i> | tooth and jaw germ cells | Jackman and Stock, 2006; Aigler et al., 2014; Li et al., 2016 |
| <i>tg(ela:eGFP)</i> | pancreatic cells | Wan et al., 2006; Calzolari et al., 2014; Navis and Bagnat, 2015 |
| <i>tg(fli1-eGFP)</i> | endothelial and haematopoietic cells | Lawson and Weinstein, 2002 |
| <i>tg(gata1:eGFP)</i> | blood cells | Traver et al., 2003a,b |
| <i>tg(gata2:eGFP)</i> | blood cells | Long et al., 1997 |
| <i>tg(hsp70l:canotch3-eGFP)</i> | perivascular system | Wang et al., 2014a,b |
| <i>tg(hsp70l:shha-eGFP)</i> | retina | Ertzer et al., 2007; Shen et al., 2013 |
| <i>tg(ins:GFP)</i> | insulin/pancreatic cells | Huang et al., 2001; Li et al., 2009; Eames et al., 2013 |
| <i>tg(kdr:eGFP)</i> | angioblasts and endothelial precursor cells | Cross et al., 2003; Jin et al., 2006 |
| <i>tg(l-fabp:DBP-EGFP)</i> | liver and vasculature | Her et al., 2003a,b; Xie et al., 2010 |
| <i>tg(mpeg1:eGFP)</i> | macrophage lineage | Ellett et al., 2011; Fang et al., 2011; Hall et al., 2013; Brudal et al., 2014, 2015 |
| <i>tg(mpx:eGFP)</i> | neutrophil lineage | Renshaw et al., 2006; Ellett et al., 2011; Benard et al., 2012; Wang et al., 2014a; Shen et al., 2016 |
| <i>tg(my17:eGFP)</i> | myocardial cells | Ho et al., 2007 |
| <i>tg(nkx2.3:efnb2a,myl7:eGFP)</i> | arterial cells | Choe and Crump, 2015 |
| <i>tg(pax2a:GFP)</i> | optic vesicle and epibranchial placodes | McCarroll et al., 2012; Aguillon et al., 2016; Juarez-Morales et al., 2016 |
| <i>tg(scl-α:dsRED)</i> | intermediate endothelial cells | Zhen et al., 2013 |
| <i>tg(scl-β:d2eGFP)</i> | anterior-posterior endothelial cells | Zhen et al., 2013 |
| <i>tg(Tie2:eGFP)</i> | endothelial cells | Motoike et al., 2000 |
| <i>tgBAC(cdh5:Citrine)</i> | pan-endothelial cells | Bussmann and Schulte-Merker, 2011; Bussmann et al., 2011 |
| <i>tgBAC(dll4:GAL4FF)</i> | endothelial cells | Hermkens et al., 2015 |
| <i>tgBAC(flt4:Citrine)</i> | pan-endothelial cells | Gordon et al., 2013 |

tg(mpx:eGFP), expresses GFP under the neutrophil-specific myeloperoxidase promoter (Renshaw et al., 2006). A macrophage-specific marker, *mpeg1* was used to drive eGFP in the zebrafish line *tg(mpeg1:eGFP)* (Ellett et al., 2011). Combining transgenic lines of zebrafish with transparent strains (ie Casper mutant zebrafish) can enable the observation and analysis of the interactions between cancer cell and its living environment.

Targeted mutagenesis

A major breakthrough in zebrafish gene targeting was the creation of synthetic transcription activator-like effector nucleases (TALENs). TALENs can induce a DNA break at virtually any desired spot in the genome (Bogdanove and Voytas, 2011; Mussolino and Cathomen, 2012; Zu et al., 2013). The use of TALENs made it relatively simple to quickly create zebrafish models that epitomize the flaws of biochemical pathway, which contribute to various disease states. It was thought that TALENs may be of use in drug discovery, as well as the study of gene regulation. However, overall difficulties with design, synthesis, and validation has limited the widespread adoption of TALENs for routine use (Gupta and Musunuru, 2014). The development of the CRISPR/Cas9 system (Jinek et al., 2012; Cho et al., 2013; Cong et al., 2013; Mali et al., 2013) as a new tool used to edit the genome of living cells is revolutionising molecular biology.

CRISPR/Cas9 zebrafish

CRISPR/Cas9 is a new technology that has profoundly changed targeted mutagenesis. It is based on bacterial CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats) and CRISPR-associated (Cas) genes, this method of genetic engineering produces similar efficiencies to TALENs (Hwang et al., 2013), but with a more robust and easier to use technology.

Briefly, the CRISPR/Cas9 technology consists of a synthetic guide RNA (gRNA) and Cas9 protein that work in concert with one another. The guide RNA is designed to target the gene of interest. This synthetic RNA *guides* the Cas9 (an endonuclease enzyme) to the target DNA. Cas9 then acts as a *molecular scissor* and cuts/edits that targeted DNA. A good review of the general CRISPR/Cas9 techniques and history was published earlier this year in Bioassays (Stella and Montoya, 2016). In zebrafish, the CRISPR/Cas9 technology is operated by directly injecting the gRNA and Cas9 mRNA into a one-cell stage embryo (Hruscha et al., 2013; Hwang et al., 2013; Jao et al., 2013). Multiple gene knock-out or large chromosomal deletions are also possible with this technique (Xiao et al., 2013). Of note, the mutations generated by the CRISPR/Cas9 technology are inherited by the next generation of zebrafish - thus creating stable knock-out zebrafish lines

is theoretically rapid and simple (Talbot and Amacher, 2014). Knock-in strategies have also been developed using the CRISPR/Cas9 system (Chang et al., 2013; Hruscha et al., 2013; Auer et al., 2014a,b; Irion et al., 2014; Kimura et al., 2014).

Earlier this year, Schier et al (McKenna et al., 2016) used CRISPR/Cas9 in embryonic zebrafish in a breakthrough study of developmental biology. They developed a technique, GESTALT (Genome Editing of Synthetic Target Arrays of Lineage Tracing), to progressively introduce, and thus accumulate, a wide range of mutations over several rounds of cell divisions. The rate and pattern of the edits were analysed and by sampling a large number of cells from various organs in the zebrafish the group were able to monitor which cells developed certain mutations over time. By analysing the number and type of mutations in each cell, they were able to conclude that most cells in the adult zebrafish's organs were derived from only a few embryonic progenitors. GESTALT has immediate applications for developmental biologists, but, this technique may also be applied to cancer research, including a better understanding of cancer initiation, and metastatic tumor sites in relation to the initial tumor.

The importance of targeted mutagenesis in zebrafish should not be ignored. Systems such as TALENs and CRISPR/Cas9 will make it possible to modify the microenvironment of the xenografted tumour. Furthermore, by knocking-in certain genes (e.g. human specific cytokines, receptors, and growth factors) it may be possible to make the zebrafish more human. Such humanization of a host environment has already been used with mouse xenograft models (Wunderlich et al., 2010; Willinger et al., 2011).

Interactions with the tumor microenvironment

Zebrafish xenotransplantation enables direct observation of the communication between transplanted cancer cells and their microenvironment. The developing tumour has a microenvironment comprising of the tumor stroma, blood vessels, inflammatory cells and an assortment of other related tissue cells, as well as the proliferating cancer cells (Whiteside, 2008). It is an inimitable environment that develops as a result of the interactions of the tumour with the host. It is the cancer which coordinates the different events (molecular and cellular) taking place in neighbouring tissues. A limitation of studying the microenvironment is that the communication mechanisms may not be active across species. However, studies have shown that ligands secreted by human tumor cells are active on zebrafish host cells, and likewise, zebrafish ligands are active on human cells (Lee et al., 2005; Topczewska et al., 2006; Tulotta et al., 2016).

One of the first studies to look at the tumor microenvironment was performed by Lee et al. (2005). Human metastatic melanoma cells that exhibited a dedifferentiated phenotype were transplanted into

blastula-stage embryos to understand the developmental flexibility in response to signals from the micro-environment. These human metastatic melanoma cells survived, exhibited motility, and divided. These cells maintained a state of dedifferentiation, did not form tumors, nor integrate into the host organs. This is vastly different from what is seen when normal human melanocytes are transplanted - the normal cells were frequently found disseminated throughout the microenvironment of the skin, as expected. Thus, the homing cues present in a zebrafish embryo can be interpreted by normal human cells. Another study has shown that host development is influenced by tumor cells (Topczewska et al., 2006). Topczewska et al have shown that an embryo transplanted with melanoma cells will ectopically form the embryonic axis. Their analysis discovered that inhibition of Nodal, a potent embryonic morphogen that is secreted by melanoma cells, promoted the return of a melanocytic phenotype in melanoma cells and diminished the tumorigenicity of these cells. These results not only suggested the importance of Nodal signalling in the progression of melanoma, but provided another promising therapeutic target for melanoma.

Recently, zebrafish were used to demonstrate cross-communication between host and human ligands and receptors. The conserved communication between human breast cancer cells expressing CXCR4 and the zebrafish host was exploited by the CXCR4 antagonist, IT1t, and genetic inhibition of CXCR4 signalling. Importantly, human cells expressing CXCR4 initiated the early metastatic events by detecting similar zebrafish ligands at the metastatic site. Both the tumor size and spread were significantly reduced in the zebrafish mutants of cxcl12 ligands, and direct activation of human CXCR4 receptors on tumor cells by cxcl12 was demonstrated (Tulotta et al., 2016).

Angiogenesis

Angiogenesis is an important promoter of tumor growth, invasion, and metastases, and is a potential therapeutic target (reviewed in Mittal et al., 2014). The characteristics of the zebrafish can be exploited to investigate tumor angiogenesis. Development of new tools and techniques, including genetic manipulation and *in vivo* imaging techniques has advanced the study of angiogenesis in zebrafish models (Tobia et al., 2011). Confocal microangiography is a technique for visualizing patent blood vessels and has been used in developing zebrafish (Cheng et al., 2001). Furthermore, imaging chambers have been developed to deliver the continuous circulation of warm oxygenated aqueous media (Kamei and Weinstein, 2005). This chamber has been used on *tg(fli1a-eGFP)* zebrafish for 5 days of multiphoton time-lapse imaging to visualise the developing blood vessels in the body. This technique did not interfere with development or viability, nor was there evidence of strength reduction of the circulatory flow.

Recently, a group looked for old drugs with

antiangiogenic activity using the zebrafish model (Zhu et al., 2016). The U.S. Drug Collection Library was screened in the transgenic *tg(fli1a-eGFP)* zebrafish line and 11 old drugs with antiangiogenic activity were identified. One drug, Closantel, was verified as having antiangiogenic activity in zebrafish by examining the intersegmental and subintestinal vessels. Furthermore, Closantel significantly repressed the growth of xenotransplanted human cancer cell lines, in a dose-dependent manner. The study used human lymphoma, cervical cancer, pancreatic cancer, and liver cancer cells.

Another recent study used the zebrafish model to investigate glioma stem cells (GSCs) - looking at tumor angiogenesis, invasion, and proliferation (Yang et al., 2014). The researchers determined that some previously verified anti-angiogenic agents repressed xenografted-GSCs induced angiogenesis. Nordy, the synthetic dl-nordihydroguaiaretic acid compound (dl-NDGA), was further analysed and was shown to reduce GSCs invasion and proliferation via the regulation of the arachidonate 5-lipoxygenase (Alox-5) pathway. Interestingly, an enhanced suppression of GSC-induced-angiogenesis can be achieved through the combination of Nordy and a vascular endothelial growth factor (VEGF) inhibitor.

The zebrafish/tumor xenograft angiogenesis assay has been used to not only look at anti-angiogenic drugs, but also microRNAs (Chiavacci et al., 2015). A prostate cancer cell line, DU-145, has been transfected with four microRNAs responsive to both anti- and pro-angiogenic stimuli. After transfection, the DU-145 cells were transplanted into *tg(Kdrl:eGFP)s843* zebrafish embryos, near the developing subintestinal vessels. The *tg(Kdrl:eGFP)s843* line of zebrafish have been used previously in the study of vascular development (Jin et al., 2005). At 72 h post-fertilization the GFP-positive vessels permeated through the graft of DU-145 transfected with 3 of the microRNAs. However, vessel formation and tumor cell invasion was inhibited with the DU-145 cells transfected with remaining microRNA. These results show that the zebrafish/tumor xenograft is a model that is useful for the study of angiogenesis. This model identified the microRNAs needed to suppress the release of angiogenic growth factors by tumor cells.

Syngeneic zebrafish

Syngeneic zebrafish have greatly assisted with tumor transplantation studies (Mizgirev and Revskoy, 2006, 2010; Smith et al., 2010). Syngeneic, or allograft, models were developed over 50 years ago as a murine tumour model. Syngeneic models retain an intact immune system, and are therefore exceptionally relevant for studies of immunologically-based targeted therapies (Dranoff, 2012). Of note, syngeneic zebrafish have been used to study self-renewing cancer cells - the only cell types within a tumor that have an unlimited ability to

promote cancer growth. The hypothesis underlying these experiments is: targeting self-renewing cells will impede tumor progression and prevent relapse, thus bettering the patient's prognosis (Zhou et al., 2009). The most frequent way to establish the incidence of self-renewing cells within a tumor is with a limiting dilution cell transplantation assay (Dick et al., 1997; Tatekawa et al., 2006). Briefly, the cancer cells are xenotransplanted into several host animals at increasing doses. The ratio of animals that develop tumours are used to gauge the number of self-renewing cells from the original tumor sample. Such a large scale experiment involving mice is expensive, thus most experiments on limiting dilution assays use 10-15 mice (Blackburn et al., 2011). A method has been developed where fluorescently labelled cancer cells can be transplanted into adult fish in order to determine the number of self-renewing cells (Blackburn et al., 2011). T-cell acute lymphoblastic leukaemia was provided as the example, although the protocol can be adapted to ascertain the number of self-renewing cells using any cancer model in the zebrafish. To date, this model has been used in T-cell acute lymphoblastic leukaemia (Smith et al., 2010) and rhabdomyosarcoma (Ignatius et al., 2012). Importantly, this model has been successfully used to transplant single cells (Smith et al., 2010). This model is perfect for limiting dilution transplantation assays, as the cancer cells do not need to undergo adaption for growth in a foreign micro-environment, which could underestimate the frequency of cell self-renewal (Kelly et al., 2007; Rosen and Jordan, 2009).

Limitations

The characteristics of the zebrafish xenograft model provide a robust model for the study of human cancer, but it is not without its limitations. As described earlier, human and zebrafish genomes are similar (Schartl, 2014). However there are several cancer-associated genes not expressed in zebrafish, including BRCA1, p16, and IL6 (Howe et al., 2013). Not only does this present a challenge when investigating the function of these omitted genes, or their pathways, but the molecular mechanisms linking the host to the cancer cells may not be conserved either. Thus, the communication between the human tumor cells and the zebrafish micro-environment may be impacted. In the future, it may be possible to humanize the zebrafish model. Mouse models have been humanized for the study of infectious disease (Brehm et al., 2013)

The zebrafish xenograft models also rely on an impaired adaptive immune system. The absence of an adaptive immune response is advantageous for the initial transplantation and injection of human cells, this benefit may become a limitation when translating the findings. The adaptive immune cells can play an essential role in the promotion or inhibition of cancer growth and development and are also involved in the effects of certain cancer treatments (Morgan, 2012; Gajewski et

al., 2013).

There are also limitations with the different physiological requirements of using a zebrafish host and human cancer cells. Zebrafish embryos are routinely maintained at 28°C, and mammalian (including human) cells are grown at 37°C. Maintaining mammalian cells at 28°C does not signify an optimal temperature for cell growth and metabolism (Nicoli et al., 2007). As a compromise, post-xenografted embryonic zebrafish are housed at 32-35°C, with no obvious effects on zebrafish development (Haldi et al., 2006). The increase in the temperature of the zebrafish environment is hyperthermic and could be a source of stress (Rey et al., 2015). In contrast, these temperature are considered mildly hypothermic for mammalian cells. The effects of mild hypothermia on the growth and metabolism of Chinese hamster ovary (CHO) cells was recently studied (Vergara et al., 2014). Mammalian cells grown at 33°C showed a slower growth rate and differences in metabolite production.

Finally, a xenograft does not portray the complexity of a human tumor. The majority of human cancers take years to grow. Tumor xenografts in a mouse model are designed to grow in weeks, not years, and tumor xenografts in an embryonic zebrafish model are designed to grow in days, not weeks. There have been several studies that analysed the considerable differences between primary tumours and established cell lines. The changes in the genomic stability and gene expression are of particular importance (such as Lee et al., 2006), and this is another limitation of using a model dependent on cell lines. Although primary tumor cells can be implanted into zebrafish, as described earlier, this is still far removed from the developmental process of a human tumor.

Future perspectives

Tumor organoids

The evidence presented in this review show that the zebrafish xenograft model offers many experimental strengths that work well alongside other models. One such model that is gaining momentum is the tumor organoid. Tumor organoids are 3D cultures of tumor cells that have self-organized and partially reproduce the complexity of a tumor (van de Wetering et al., 2015). Organoids fall in an area between the cancer cell lines and patient derived tumor xenografts (PDTX) (Sachs and Clevers, 2014). Current short-comings with organoid cultures, such as lacking a microenvironment are being addressed in the field (Huch and Koo, 2015). One possible outcome of the organoid is a patient-derived organoid that could be used to directly test the drug sensitivity of the cancer as part of a personalised treatment system (Clevers, 2016). Furthermore, novel compounds could also be tested in a similar manner for clinical research. Provided that drugs are optimized for favourable pharmacokinetic and pharmacodynamics

properties and have a good safety profile, we would propose that when positive results on a particular targeted therapy are obtained in both zebrafish xenograft and tumor organoid models, this would provide sufficient evidence to test the novel drug, (or combination of drugs) in cancer patients as part of early phase clinical trials.

Experimental automation and rapid high-resolution imaging

Zebrafish xenotransplantation is progressing towards enhanced experimental automation and fast high-resolution imaging. Most xenotransplantation studies manually inject the human cells into the zebrafish embryo - which can be both laborious and technically challenging. Methods have been developed where a robot injects zebrafish embryos with a speed of up to 2000 embryos per hour with very high accuracy (Spaink et al., 2013). Tests using this injection robot have used the highly aggressive osteosarcoma cell lines SJSA-1, osteosarcoma line L2792, the human prostate cells PC3 and LNCAP, and the melanoma cell line Mel57-VEGF. The automated injection of cells into the yolk sac occurred at the early pre-gastrulation stage zebrafish (256-cell stage). Depending on the cancer cell type used, Spaink et al have demonstrated the growth and invasion of the xenografted cells into the tail fin. Continuing on from this technique, an arrangement whereby the robotic injection system is subsequently imaged via high-throughput technologies (Ghotra et al., 2012) has been used as a screen for many different mammalian cancer cell lines in the zebrafish embryo model. The Complex Object Parametric Analyzer and Sorter (COPAS, Union Biosciences) is a flexible platform that is amenable to the use of automatically sorting zebrafish embryos. COPAS allows embryos to be selected on the basis of a number of parameters, including length, optical density, or fluorescence expression. Thus, COPAS can be adapted to measure cancer cell proliferation. High-throughput imaging systems can be used before and after injection of fluorescent cells, and also in chemical screens (Letamendia et al., 2012; Mathias et al., 2012). This is possible because of the zebrafish's small size, transparency, number of offspring, and the ability to grow in a liquid medium. The combination of an automatic injection step with subsequent COPAS sorting provides a mighty *in vivo* platform for the study of human cancer. This automated approach could be used to study patient-derived specimens.

Preclinical and clinical applications

The zebrafish xenograft model can be used to test experimental anticancer drugs. This is often quite simple as the drugs need only be added to the fish water. For example, quisinostat (a histone deacetylase inhibitor) and MLN-4924 (a neddylation pathway inhibitor) were assessed in a zebrafish uveal melanoma xenograft model

(van der Ent et al., 2014a). After these drugs were directly added to the water of uveal melanoma-engrafted embryos, the growth and invasion of the cells was quantified using an automated confocal image analysis platform. Both drugs were shown to block migration and proliferation of the cancer cells.

Another excellent example used the CXCR4 inhibitor, IT1t (Tulotta et al., 2016). The CXCR4-CXCL12 chemokine signalling axis helps to direct cell migration in both physiological and pathological conditions. As a potential target for the highly aggressive triple-negative breast cancer subtype, Tulotta et al used a zebrafish xenograft model and successfully blocked early metastatic events with the CXCR4 inhibitor. Not only did this study propose a pharmacological approach to targeting triple-negative breast cancer, but also validated the zebrafish xenograft model.

Primary human tumors, such as pancreatic, colon, stomach (Marques et al., 2009), prostate (Bansal et al., 2014) and primary leukaemia (Pruvot et al., 2011) samples, were injected into zebrafish embryos, where they proliferated and spread throughout the host. Furthermore, a study was recently published describing the development of a patient-derived xenograft of breast cancer bone metastasis (Mercatali et al., 2016). Zebrafish offer a key benefit when working with precious biopsy samples compared to larger animal models - typically zebrafish xenografts will require only 100–200 cells per zebrafish embryo. Furthermore, because the engraftment does not require a lot of time, it could be used as a tool for predicting patient response to treatments. Potentially, tissues or cells from a patient could be transplanted into hundreds of zebrafish embryos and the response to the different available drug treatments would be monitored. This would yield constructive information regarding the most suitable drugs administered for each individual patient.

Conclusions

The unique attributes of zebrafish are being increasingly recognised in cancer biology. The zebrafish model offers a fast, cheap, and powerful means of investigating human cancer. Advances in genetic manipulation, automated injection, and imaging techniques have provided researchers with an unprecedented opportunity to use zebrafish as a tool to understand the many different types of human cancer and their ability to proliferate, invade, metastasise, and communicate with the microenvironment. The zebrafish is not a complete model for human cancer - it has several limitations. However, zebrafish xenografts can be used to study the molecular pathways used in cancer cells. Zebrafish embryos can be used to study the micro-environment and angiogenesis. Furthermore, this model can be utilised as a tool for predicting patient responses to drug therapies, as well as being a simple model that is suitable for high-throughput screening, and it works well in conjunction with more complex models. The zebrafish

proves a rapid, robust, and inexpensive model for the study of human cancers.

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